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Plum Pox Virus RT-PCR Detection Kit

Product Insert

Product # 33400

Pathogen Information

Plum pox virus (PPV) is a member of the genus *Potyvirus*, and the virus is considered the most serious pathogen affecting stone fruits including apricots, cherry, nectarine, peaches, and plums. The symptoms of plum pox disease or Sharka include fruit abnormality, loss of taste, and fruits that drop prematurely. These symptoms may cause losses as high as 80–100% of a crop and consequently it is a disease of economic significance in every country where stone fruits are grown. To achieve successful eradication or effective management of the associated disease, sensitive detection of the virus in infected plants is required.

Principle of the Test

Norgen's Plum Pox Viurs (PPV) RT-PCR Detection Kit constituents a ready-to-use system for the isolation and detection of PPV using end-point one step RT-PCR. The kit first allows for the isolation of PPV RNA from plant tissues using spin-column chromatography based on Norgen's proprietary resin. The RNA is isolated free from inhibitors, and can then be used as the template in a one step RT-PCR reaction for PPV detection using the provided PPV Master Mix. The PPV Mastermix contains reagents and enzymes for the specific amplification of a 295 bp region of the virus genome. In addition, Norgen's PPV RT-PCR Detection Kit contains a second Mastermix, the Control 2X RT-PCR Master Mix, which can be used to identify possible PCR inhibition and/or inadequate isolation via a separate RT-PCR reaction with the use of the provided *PCR control (PCRC)* or *Isolation Control (IsoC),* respectively. This kit is designed to allow for the testing of 24 samples.

Kit Components:

Component	Contents
Lysis Solution	25 mL
Wash Solution	11 mL
Elution Buffer	2 mL
Mini Spin Columns	24
Collection Tubes	24
Elution tubes (1.7 mL)	24
PPV 2x RT-PCR Master Mix	0.35 mL
Control 2x RT-PCR Mastermix	0.35 mL
Isolation Control (IsoC)*[a]	0.3 mL
PPV Positive Control (PosC)*[b]	0.1 mL
Nuclease Free-Water	1.25 mL
Norgen's DNA Marker	0.1 mL
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** IsoC = Isolation Control ; PosC= Positive Control*
[a] The isolation control is a RNA transcript product.
[b] The positive control is PPV RNA transcript

Customer-Supplied Reagents and Equipment

- Benchtop microcentrifuge
- 1.5 mL microcentrifuge tubes
- 96 – 100% ethanol
- 70% ethanol
- Mortar and pestle or other homogenization device

Storage Conditions and Product Stability

- The Positive Control (**PPV *PosC,*** red cap) and Isolation Control (***IsoC*,** orange cap) should be stored at -70°C. If needed, make aliquots of the controls according to the volume used in the protocol (10 µL of **PPV *PosC*** or 10 µL of ***IsoC***) prior to freezing.
- The **PPV 2X Detection RT-PCR Mastermix** (green cap) and the **Control 2X RT-PCR Mastermix** (yellow cap) should be stored at -20°C upon receipt (-70°C for long-term). Make appropriate aliquots and store at -20°C if needed.
- All other kit components may be stored at room temperature
- The **PPV 2X Detection RT-PCR Mastermix** and the **Control 2X RT-PCR Mastermix**, Positive Control and Isolation Control should not undergo repeated freeze-thaw (a maximum freeze-thaw of three times).
- For RT-PCR
    - Allow reagents to thaw at room temperature prior to use
    - When thawed, mix the components and centrifuge briefly
    - Work quickly on ice
    - After addition of RT-PCR Mastermix use within one hour

General Precautions

The user should exercise the following precautions when using the kit:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's PPV RT-PCR Detection Kit, including the PPV 2x RT-PCR Master Mix, Control 2X RT-PCR Mastermix, Isolation Control and PPV Positive Control are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's PPV RT-PCR Detection Kit is designed for research purposes only.

Product Warranty and Satisfaction Guarantee

NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Disclaimers

The **Lysis Solution** contains guanidinium salts, and should be handled with care. Guanidinium salts form highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions.

Safety Information

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Working with RNA
RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations.
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination.
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only.
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water.
- Clean all surfaces with commercially available RNase decontamination solutions.
- When working with purified RNA samples, ensure that they remain on ice during downstream applications.

Protocol

A. PPV Total RNA Isolation

Important Notes Prior to Beginning Protocol:

- All centrifugation steps are carried out in a benchtop microcentrifuge at **14,000 x g (~ 14,000 RPM)** except where noted. All centrifugation steps are performed at room temperature.
- A variable speed centrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Ensure that all solutions are at room temperature prior to use.
- Prepare a working concentration of the **Wash Solution** by adding 25 mL of 95 - 100% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 36 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Both fresh or frozen samples may be used for this procedure. Samples should be flash-frozen in liquid nitrogen and transferred immediately to a -70°C freezer for long-term storage. Do not allow frozen samples to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.
- While the provided procedure does not rely on the use of liquid nitrogen to homogenize the sample, both fresh and frozen tissues can optionally be processed using other homogenization methods, including grinding with liquid nitrogen.
- **Isolation Control (*IsoC*)**
    - An Isolation Control (*IsoC*) is supplied. This allows the user to control the RNA isolation procedure. For this assay, add the Isolation Control *(IsoC)* to the lysate during the isolation procedure
    - The Isolation Control *(IsoC)* must not be added to the sample material directly.
    - Do not freeze and thaw the Isolation Control *(IsoC)* more than 2 times.
    - The PPV Isolation Control *(IsoC)* must be kept on ice at all times during the isolation procedure.
- The RT-PCR components of the PPV RT-PCR Detection Kit should remain at -20°C until RNA is extracted and ready for RT-PCR amplification.
- It is important to work quickly during this procedure.

1. Lysate Preparation

a. Transfer ≤100 mg of plant tissue into a mortar that contains 800 µL of **Lysis Solution**. (The volume of plant tissue and Lysis Solution can be increased proportionally. For instance, 0.5g of plant tissue requires 4 mL of **Lysis Solution**. Extra lysis solution can be purchased separately. See Related Products table). Grind the sample using a pestle until the tissue is completely macerated.

Note: Other homogenization devices such as Bioreba extraction bag and a homogenizer can also be applied to this procedure.

b. Using a pipette, transfer the lysate into an RNAase-free microcentrifuge tube (not provided).
c. Spin the lysate for 2 minutes to pellet any cell debris. Transfer the supernatant to another RNase-free microcentrifuge tube. Note the volume of the supernatant/lysate.

Note: Ensure that only the clear supernatant is transferred, avoiding any of the debris. If necessary, repeat Step **1c** if visible precipitates are still present after the first spin.

d. Add an equal volume of 70% ethanol (provided by the user) to the lysate collected above (100 µL of ethanol is added to every 100 µL of lysate). Vortex to mix. **Proceed to Step 2.**

2. Binding RNA to Column

a. Assemble a column with one of the provided collection tubes.
b. Add 10 µL of **Isolation Control (*IsoC*)** to the lysate mixture.
c. Apply up to 600 µL of the clarified lysate with ethanol onto the column and centrifuge for 1 minute at **14000 × g (~14,000 RPM**). Discard the flowthrough and reassemble the spin column with the collection tube.

Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.

d. Depending on your lysate volume, repeat step **2c** if necessary.

3. Column Wash

a. Apply 400 µL of **Wash Solution** to the column and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flowthrough and reassemble the spin column with its collection tube.
c. Repeat steps **3a** and **3b** to wash column a second time.
d. Wash column a third time by adding another 400 µL of **Wash Solution** and centrifuging for 1 minute.
e. Discard the flowthrough and reassemble the spin column with its collection tube.
f. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.

4. RNA Elution

a. Place the column into a fresh 1.7 mL Elution tube provided with the kit.
b. Add 50 µL of **Elution Buffer** to the column.
c. Centrifuge for 2 minutes at **200 x *g* (~2,000 RPM)**, followed by a 1 minute spin at **14,000 x *g* (~14,000 RPM)**. Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at **14,000 x g (~14,000 RPM)** for 1 additional minute.
d. The purified RNA sample could be used immediately for RT-PCR as described below. It is recommended that samples be placed at -70°C for long term storage.

B. PPV RT-PCR Assay Preparation

Notes:

- Before use, suitable amounts of all RT-PCR components should be completely thawed at room temperature, gently vortexed and centrifuged briefly.
- The amount of PPV 2X RT-PCR Master Mix provided is enough for up to 32 RT-PCR reactions (24 sample RT-PCR, 4 positive control RT-PCR and 4 no template control RT-PCR).
- For each sample, one RT-PCR reaction using the **PPV 2X Detection RT-PCR Mastermix** and one RT-PCR reaction using **Control 2X RT-PCR Mastermix** should be set up in order to have a proper interpretation of the results.
- For every RT-PCR run, one reaction containing PPV Positive Control (**PPV *PosC***) and one reaction as no template control (*Nuclease Free-Water*) must be included for proper interpretation of results.
- The recommended minimum number of RNA samples tested per RT-PCR run is 6.
- Using a lower volume from the sample than recommended may affect the sensitivity of PPV Limit of Detection.

1. Prepare the RT-PCR reaction for sample detection (Set #1, using **PPV 2X Detection RT-PCR Mastermix**) and the RT-PCR reaction for control detection (Set #2, using **Control 2X RT-PCR Mastermix**) as shown in Table 1 below. The recommended amount of sample RNA to be used is 1 - 2 µL. Ensure that one PPV detection reaction and one control reaction is prepared for each RNA sample. Adjust the final volume of the RT-PCR reaction to 20 µL using the Nuclease-Free Water provided.

Table 1. RT-PCR Assay Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
PPV 2X RT-PCR Master Mix Or Control 2X RT-PCR Master Mix	**10 µL**
Sample RNA	**2 µL**
Nuclease-Free Water	**8 µL**
Total Volume	**20 µL**

2. For every RT-PCR run, prepare **one** positive control RT-PCR as shown in Table 2 below:

Table 2. RT-PCR Positive Control Preparation

RT-PCR Components	Volume Per RT- PCR Reaction
PPV 2X RT-PCR Master Mix Or Control 2X RT-PCR Master Mix	**10 µL**
PPV *Positive Control (PosC)*	**10 µL**
Total Volume	**20 µL**

3. For every RT- PCR run, prepare **one** no template control RT-PCR as shown in Table 3 below:

Table 3. RT-PCR Negative Control Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
PPV 2X RT- PCR Master Mix Or Control 2X RT-PCR Master Mix	**10 µL**
Nuclease-Free Water	**10 µL**
Total Volume	**20 µL**

Therefore, at a minimum, each PCR run will contain 6 separate RT-PCR reactions.

C. One Step RT- PPV PCR Assay Programming

1. Program the thermocylcer according to the program shown in Table 4 below.
2. Run one step RT-PCR.

Table 4. PPV Assay Program

One Step RT-PCR Cycle	Step	Temperature	Duration
Cycle 1	**Step 1**	**50°C**	**30 min**
Cycle 2	**Step 1**	**95°C**	**3 min**
Cycle 3 (40x)	**Step 1**	**94°C**	**15 sec**
	Step 2	**55°C**	**30 sec**
	Step 3	**72°C**	**45 sec**
Cycle 4	**Step 1**	**72°C**	**5 min**
Cycle 5	**Step 1**	**4°C**	**∞**

D. PPV One Step RT- PCR Assay Results Interpretation

1. For the analysis of the RT-PCR data, the entire 20 µL RT-PCR Reaction should be loaded on a 1X TAE 1.5% Agarose RNA gel along with 10 µL of Norgen's DNA Marker (provided).
2. The RT-PCR products should be resolved on the 1X TAE 1.5% Agarose gel at 150V for 20 minutes (Gel running time will be vary depending on an electrophoresis apparatus).
3. Sample results are provided below:

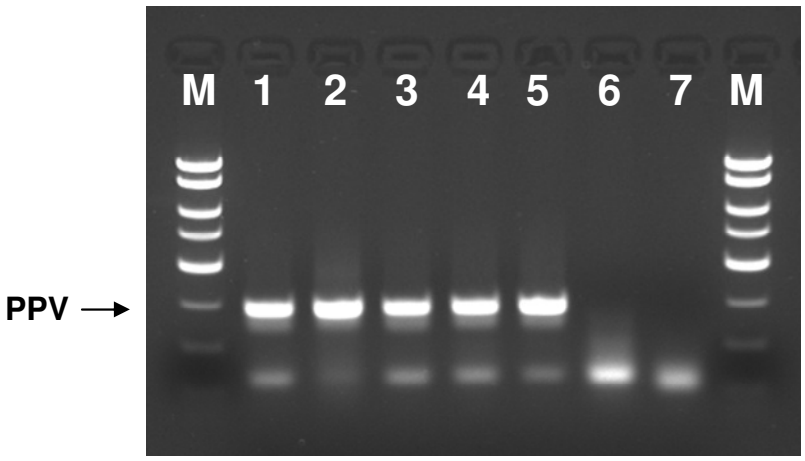

Figure 1: A representative 1X TAE 1.5% agarose gel showing the amplification of PPV negative (Lanes 6 and 7) positive (Lanes 1 to 5) controls. The size of the PPV target amplicon corresponds to 295 bp as represented by the provided DNA Marker (M).

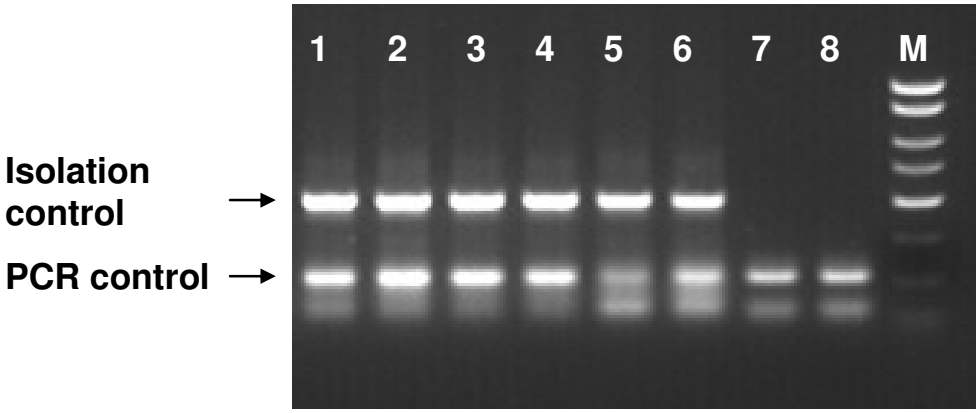

Figure 2: A representative 1X TAE 1.5% agarose gel showing the amplification of **Isolation Control** and **PCR Control** under different conditions using the **Control 2X RT-PCR Mastermix**. The size of the Isolation Control amplicon and PCR Control amplicon correspond to 499 bp and 150 bp, respectively, as represented by the provided DNA Marker (M). Lanes 1 to 6 showed detection of both Isolation Control and PCR Control, suggesting that the RNA isolation as well as the RT-PCR reaction was successful. Lane 7 and 8 showed only the detection of PCR Control suggesting that while the RT-PCR was successful, the isolation failed to recover even the spiked-in Isolation control.

Table 5. Interpretation of One Step RT-PCR Assay Results

Input Type	Target reaction	Control Reaction		Interpretation
	PPV Target Band (295 bp)	*IsoC* Band (499 bp)	*PCRC* Band (150 bp)	
Positive Control	X	X	X	Valid
Negative Control			X	Valid
Sample	X	X	X	Positive
Sample		X	X	Negative
Sample			X	Re-test
Sample				Re-test
Sample		X		Negative
Sample	X		X	Positive
Sample	X	X		Positive
Sample	X			Re-test

** For results obtained that are not covered in Table 5 above, please refer to the Troubleshooting Section.

E. PPV RT-PCR Assay Specificity and Sensitivity

- The specificity of Norgen's PPV RT-PCR Detection Kit is first and foremost ensured by the selection of the PPV-specific primers, as well as the selection of stringent reaction conditions. The PPV universal primers were checked for possible homologies to all plant viruses in GenBank published sequences by sequence comparison analysis and published PPV strains.

F. Linear Range

- The linear range of Norgen's PPV RT-PCR Detection Kit was determined by analysing a dilution series of an PPV quantification standards ranging from 100 ag to 1 pg.
- Each dilution has been tested in replicates (n = 4) using Norgen's PPV RT-PCR Detection Kit on a 1X TAE 1.5% agarose gel.
- The linear range of Norgen's PPV RT-PCR Detection Kit has been determined to cover concentrations from 100 ag to 1 ng
- Under the conditions of the Norgen's PPV RNA Isolation procedure, Norgen's PPV RT-PCR Detection Kit covers a linear range from 100 copies to 1 x 10^6 copies.

Frequently Asked Questions

1. How many samples should be included per RT-PCR run?

- Norgen's PPV RT-PCR Detection Kit is designed to test 24 samples. For every 6 samples, a non-template control (Nuclease Free Water) and a Positive Control must be included. It is preferable to pool and test 6 samples at a time.

2. How can I interpret my results if neither the PPV RT-PCR control nor the Isolation Control (*IsoC*) amplifies?

- If neither the PPV RT-PCR control nor the PPV Isolation Control (*IsoC*) amplifies, the sample must be re-tested. If the positive control showed amplification, then the problem occurred during the isolation, where as if the Positive control did not amplify, therefore the problem has occurred during the setup of the PCR assay reaction.

3. How should it be interpreted if only the PPV RT-PCR control showed amplification but neither the PPV target nor the PPV Isolation control amplified for a sample?

- This indicates a poor isolation. The isolation procedure must be repeated.

4. How should it be interpreted if only the Isolation Control (*IsoC*) was amplified in a sample?

- The sample tested can be considered as PPV negative.

5. How should it be interpreted if the PPV RT-PCR control and the PPV target showed amplification in a sample?

- The sample tested can be considered positive. It could happen when too much template was added to the reaction.

6. How should it be interpreted if only the PPV target and the PPV RT-PCR control were amplified in a sample?

- The sample tested can be considered as PPV positive.

7. How should it be interpreted if only the PPV target was amplified in a sample?

- The sample tested should be considered as PPV positive. At high PPV input, the PPV amplicon will be predominant and thus the PPV PCR control as well as the PPV Isolation control may not amplify as they compete for PCR resources.

8. How should it be interpreted if only the PPV RT-PCR control and the Isolation control showed amplification in a sample?

- The sample tested can be considered negative

9. What if I forgot to do a dry spin after my third wash?

- Your first RNA elution will be contaminated with the Wash Solution. This may dilute the RNA yield in your first elution and it may interfere with the PCR detection, as ethanol is known to be a PCR inhibitor.

10. What if I forgot to add the Isolation Control (*IsoC*) during the isolation?

- It is recommended that the isolation is repeated.

Related Products	Product #
Lysis Solution (100 mL)	25806
Plant RNA/DNA Purification Kit	24400
Plant/Fungi RNA Purification Kit	25800
Viroid RNA Purification Kit	32800
Bacterial Genomic RNA Isolation Kit	17900

Technical Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's Plum Pox Viurs (PPV) RT-PCR Detection Kit or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362 or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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